A simple and convenient procedure for the hydrogenation of lipids on the micro- and nanomole scale

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Summary A glass tube of a special design has been used as a vessel for the hydrogenation of lipids under slight excess pressure at 50°C. Methyl [1-^{14}C]linolenate was quantitatively hydrogenated to methyl stearate in less than 30 min. High yields were obtained on both the micromole scale (mean and standard deviation observed for quadruplicate analyses was 98.2 ± 4.8%) and the nanomole scale (94.3 ± 7.0%). The applicability of the method is demonstrated by radio-gas–liquid chromatographic analyses of nanomole amounts of ^{14}C-labeled fatty acid methyl esters from photosynthetic tissue analyzed before and after hydrogenation.

Supplementary key words radio-gas–liquid chromatography · fatty acid determination · ^{14}C-labeled fatty acids

WHEN STUDYING the composition and metabolism of lipids, and especially their fatty acids, there is often a need for hydrogenation followed by analysis of the hydrogenated products with conventional GLC or radio-GLC. There are several instruments available commercially for quantitative hydrogenation on the millimole scale and also for the qualitative hydrogenation of considerably larger samples (1–5). However, these instruments are either unnecessarily expensive if the amount of hydrogen consumed is not to be estimated, or they require samples greater than generally available in biochemical experiments. In order to overcome these problems the procedure described in this paper was developed.

Materials. Solvents were redistilled in the laboratory. Adams catalyst (platinum dioxide) was used as supplied by British Drug Houses, U.K. [1-^{14}C]Linolenic acid with a specific activity of 41.5 μCi/μmole was purchased from the Radiochemical Centre, Amersham, U.K. The methyl ester of this acid was prepared by reaction of the linolenic acid with "14% BF_3 in methanol" reagent followed by purification on thin-layer chromatography as described elsewhere (6). The activity of the methyl [1-^{14}C]linolenate isolated was about 92,000 dpm/nmole. Another aliquot of the free acid was added to a sample composed of equal parts of refined soybean oil and linseed oil. Methyl esters were then prepared by successive treatments with sodium methoxide in methanol and BF_3 in methanol (6), followed by separation of the fatty acid methyl esters by argentation thin-layer chromatography (15% $AgNO_3$ on silica gel H; Merck) in the solvent system benzene–diethyl ether 9:1 (v/v). The triene band was recovered and eluted with diethyl ether. The methyl [1-^{14}C]linolenate thus prepared had an activity of about 10,000 dpm/μmole.

Tubes of a special design, shown in Fig. 1, were manufactured at the glassblower's shop. The rubber caps, aluminium seals, and vial crimper were materials commonly used for injection vials for storage of drugs, vaccines, etc.

A Nuclear-Chicago Mark I liquid scintillation counter was used to assay the ^{14}C with a scintillator solution composed of 5 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis(2-[5-phenyloxazolyl])benzene per liter of toluene.

Radio-GLC analyses were performed on a Varian Aerograph Mod A-90-P thermal conductivity instrument coupled with a Barber-Colman series 5000 radioactivity monitor (proportional counter). The flow rate of helium was ca. 100 ml/min in the 5 ft × 1/4 inch stainless steel column packed with Chromosorb W, 80–100 mesh, containing 30% stabilized ethylene glycol adipate (Analabs, Inc., North Haven, Conn.).

Procedure. The lipid sample is evaporated in the tip of the hydrogenation tube under a stream of nitrogen. Then the tube is inverted and a slow stream of hydrogen is passed close to the tip for about 1 min. The rubber cap is inserted while the tube is still held in the inverted position, then the tube is placed in a tube rack. A small series of tubes can be filled with hydrogen consecutively and used simultaneously in one experiment. The aluminium

Abbreviations: GLC, gas–liquid chromatography.

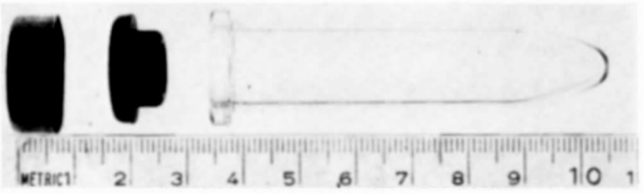

FIG. 1. Hydrogenation tube (ca. 7 cm long), with rubber septum and aluminium seal, before assembly.

seal is placed over the rubber cap and fastened to the tube with the vial crimper. This keeps the rubber cap in place when an excess pressure develops in the tube at about 50°C in a water bath. A slurry of 500 μl of methanol and about 1 mg of Adams catalyst is transferred to the tube by injection with a hypodermic syringe through the rubber septum. (It has been ascertained that the methanol does not dissolve any lipid material from the rubber cap to give "ghost" peaks on subsequent GLC recordings. Thus, the piercing of the rubber septum does not contaminate the sample.)[1] The tube is shaken vigorously (about 70 strokes/min) in a water bath kept at 50°C. After various appropriate times, generally 30 min, the tube is removed from the water bath and opened by removal of the aluminium seal.

If desired, aliquots can be taken during the hydrogenation period by transferring the tube to a centrifuge and spinning at moderate speed (ca. 2,000 *g*) for 1 min. The catalyst is thus spun down and an aliquot can be removed by a microsyringe. The hydrogenation process then can be continued and several consecutive samples can be studied.

Generally, however, the rubber cap is removed, chloroform (50 μl) is added to dissolve any lipid material poorly soluble in methanol at room temperature (e.g., very-long-chain saturated fatty acids), and the tube is centrifuged to spin down the catalyst. The clear supernatant is removed by a capillary pipette, and the catalyst is resuspended in 200 μl of chloroform. After centrifugation the supernatant solutions are pooled and the solvent is evaporated in a stream of nitrogen. The sample is dissolved in a small amount, often 25–50 μl, of hexane or carbon disulfide, and the hydrogenated methyl esters are ready for analysis by radio-GLC or conventional GLC.

Results. Previous studies had established that the samples generally were completely hydrogenated after 15 min, but since longer times were occasionally necessary, a standard time of 30 min was selected. By radio-GLC it was demonstrated that methyl stearate was the only product obtained after 30 min of hydrogenation of methyl linolenate.

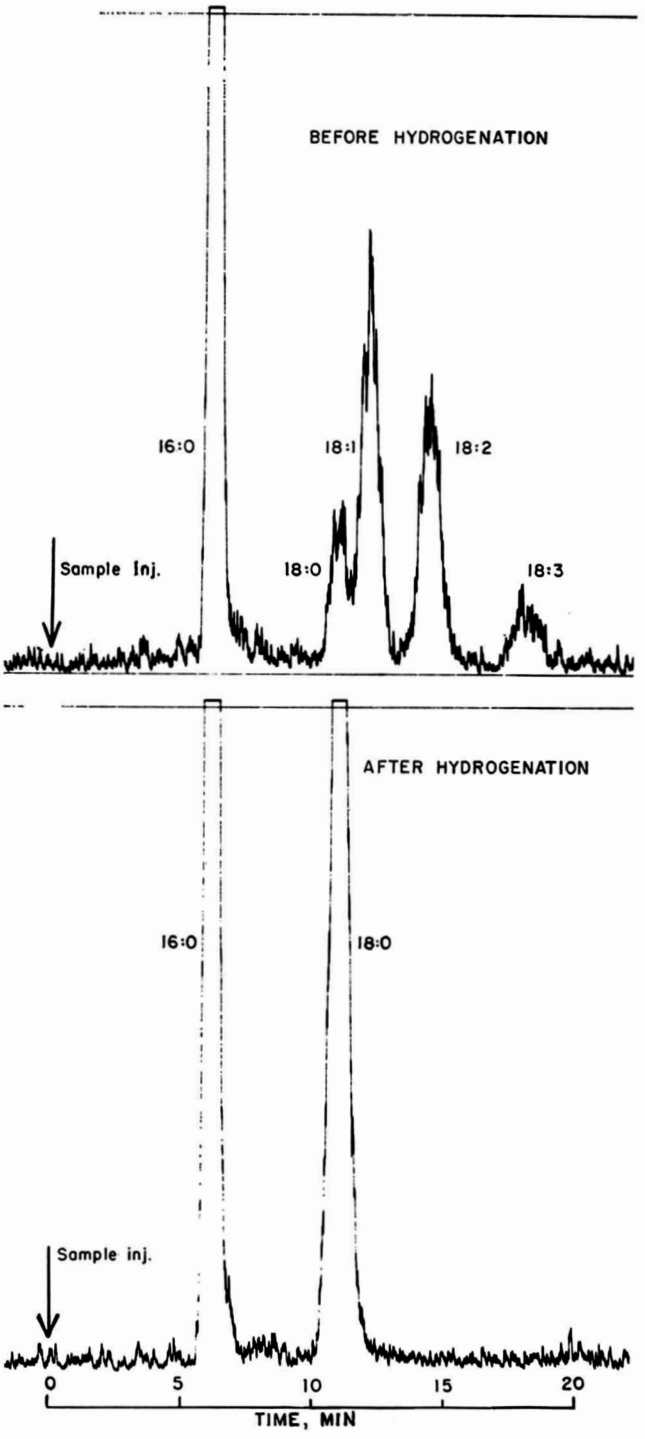

FIG. 2. Recordings of the signal from the radioactivity monitor obtained by injection of fatty acid methyl esters from ^{14}C-labeled polar lipids of *Brassica campestris* siliques.

[1] A blank sample of methanol and catalyst recently yielded some "ghost" peaks; the major one had a retention time similar to that of 19:0 on a butanediol succinate column, as determined by conventional GLC analysis. (The contaminant was not present in the methanol, but appears to be a reaction product formed by the action of the catalyst on some impurity in the methanol.) The problem was solved by using ethyl acetate as the hydrogenation solvent.

The recovery was studied in quadruplicate with the two samples of methyl [1-^{14}C]linolenate described earlier. Means and standard deviations for recovery were 98.2 ± 4.8% for the samples on the μmole scale (ca. 3 μmoles) and 94.3 ± 7.0% on the nmole scale (ca. 0.3 nmole).

When the sample to be hydrogenated cannot be freed from solvent in the hydrogenation tube either because it is more susceptible to oxidation than methyl linolenate or because it is lost by evaporation, an alternative technique can be used. The sample, preferably dissolved in methanol, is then mixed with the catalyst in a test tube and the entire slurry is injected into a hydrogenation tube that has been purged with hydrogen and closed by the vial crimper. With methyl linolenate, this alternative procedure gave only ca. 80% recovery, since minor losses occurred in each additional transfer step.

The applicability of this procedure in tracer studies is demonstrated in Fig. 2, which shows the radioactivity patterns of a sample of fatty acid methyl esters of *Brassica campestris* silique phospholipids labeled with ^{14}C by incubation of the siliques with sodium [^{14}C]acetate and analyzed before and after hydrogenation. For the hydrogenated sample a total of approximately 700 nmoles (345×10^3 dpm of ^{14}C activity) was applied in the hydrogenation experiment; a minor aliquot was used for the GLC analysis. Parallel experiments demonstrated that as little as 3700 dpm (approximately 7 nmoles) could be successfully hydrogenated and subsequently the entire sample injected into the gas chromatograph. The proportion of the area under the 16:0 peak (unchanged by hydrogenation) to that under the 18:0 peak (originally 18:0 + 18:1 + 18:2 + 18:3) was essentially the same for the 345,000 dpm sample and for the 4000 dpm sample, namely 1.00:0.67 and 1.00:0.71, respectively.

The very good recovery of added methyl [^{14}C]linolenate on the nmole scale and the simplicity and speed of the present method seem to make it a valuable tool in compositional and metabolic studies of fatty acids and acyl lipids. Most likely it is more generally applicable to hydrogenation on the nmole scale of relatively high-boiling organic compounds.

For the tracer studies, the present method should compete favorably with methods using a short precolumn, gas-phase hydrogenator in a gas chromatograph (7). This is because of both the potential danger of utilizing hydrogen as carrier gas in GLC and the frequently occurring need for further studies on the hydrogenated esters, such as determination of whether distribution of ^{14}C in the carbon chain of a fatty acid is typical for "de novo" or "elongation" mechanisms of synthesis.

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